EXHIBIT 9

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Mosquitoes can harbour yeasts of clinical significance and contribute to their environmental dissemination

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Summary

There is still a lack of studies on fungal microbiota in mosquitoes, compared with the number available on bacterial microbiota. This study reports the identification of yeasts of clinical significance in laboratory mosquito species: *Anopheles gambiae*, *Anopheles stephensi*, *Culex quinquefasciatus*, *Aedes albopictus* and *Aedes aegypti*. Among the yeasts isolated, they focused on the opportunistic pathogen *Candida parapsilosis*, since there is a need to better understand breakthrough candidaemia with resistance to the usual antifungals, which requires careful consideration in the broad-spectrum therapy, as documented in many clinical reports. *C. parapsilosis* occurs widely and has been isolated from diverse sources, including insects, which may contribute to its dissemination. In this study, it was isolated from the gut of *An. gambiae* and its presence in developmental stages and organs of different mosquito species was studied. Our results indicated that there was a stable association between *C. parapsilosis* and reared mosquitoes during the entire life cycle, and in adult male and female gut and gonads. A wide occurrence of *C. parapsilosis* was also documented in several populations of wild mosquitoes. Based on these findings, it can be said that mosquitoes might participate in the spreading of this opportunistic pathogen, not only as a carrier.

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Introduction

The yeast *Candida parapsilosis* is known as a human commensal but recently it has also been identified as an opportunistic pathogen in immunosuppressed or immunocompromised patients (Singh and Parija, 2012). Like other *Candida* species, *C. parapsilosis* is known as a saprophyte of the human digestive tract, and becomes pathogenic under specific physiologic conditions such as an imbalance of the intestinal flora. A recent study confirmed its presence in faecal and urine samples of asymptomatic donors from Bobo-Dioulasso (Burkina Faso, West Africa) (Sanata *et al*., 2014). In addition, non-*albicans* species such as *C. parapsilosis* were reported to be the most common fungal isolates from bloodstream infections in most countries of the Asia-Pacific region (Tan *et al*., 2016). This data was also confirmed in Italy, in agreement with other European reports that documented *C. parapsilosis* as one of the most frequent fungal species, responsible for more than 60% of non-*albicans* infections (Caggiano *et al*., 2015).

Notably, an outbreak of candidaemia caused by fluconazole resistant *C. parapsilosis* strains has been recently reported in intensive care units in Brazil (Pinhati *et al*., 2016). The frequent occurrence of fungal species with intrinsic resistance to the usual antifungals and which could become pathogenic at any time, should be taken into account in the therapeutic management of patients in a given geographic area (Cuervo *et al*., 2016).

In this context, it would be worth investigating which environmental factors may favour the dissemination of opportunistic pathogens. Interestingly, *C. parapsilosis* was also recovered also from non-human sources such as domestic animals (Santin *et al*., 2013) and insects, where it was previously described in the larvae of ants (Ba *et al*., 1995), in the alimentary tract of beetles (Suh *et al*., 2008) and in wild *Culex pipiens* mosquitoes from South Africa (Steyn *et al*., 2015). Mosquitoes may play an important role in the dissemination of opportunistic pathogens, especially those species whose life cycle is strictly dependent on anthropized environments.

Here we report the results of an investigation of the fungal community in different mosquito species of public health significance, focusing on *C. parapsilosis*. Five species from our insectary were investigated: *Anopheles*

gambiae and *Anopheles stephensi*, which are the major malaria vectors in Africa and Asia respectively; *Culex quinquefasciatus*, which transmits lymphatic filariasis and West Nile fever; and *Aedes albopictus* and *Aedes aegypti*, which are vectors of yellow fever, dengue, chikungunya and zika virus.

Four fungal species were identified in the gut of adult female mosquitoes by culture dependent assay: *Meyerozyma guilliermondii*, *Rhodotorula glutinis*, *Sporobolomyces cf. roseus* and *C. parapsilosis*. The literature describes these fungi as pathogens of clinical significance and suggests that their metabolic and/or antimicrobial features may indicate a possible symbiotic function in insects.

Considering the wide diffusion of the yeasts isolated in the mosquitoes, the rearing water used at insectary was analysed to evaluate which fungi could be detected in the mosquito gut as environmental contaminants. Among the isolated species, the one that showed the highest frequency in the larval water was *C. parapsilosis*. To better characterise its association with mosquitoes, the presence of *C. parapsilosis* in different organs and developmental stages of laboratory reared specimens and its occurrence in wild mosquitoes from four continents were investigated by PCR assays.

Experimental procedures

Laboratory reared mosquitoes

Mosquito samples were obtained from five colonies reared at the insectary of the University of Camerino (Italy): *An. stephensi*, *An. gambiae*, *Cx. quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus*. They were maintained at standard settings of 30°C and 80% ± 5% humidity, in axenic conditions during both the developmental and adult stages. After egg hatching, larvae were grown in clean basins filled with breeding water containing sterile minced commercial mouse food. After rinsing, the pupae were transferred to clean dishes filled with sterile water and maintained in separate cages until adult emergence. Newly emerged mosquitoes fed on Whatman paper soaked in sterilized 5% sucrose solution. Larvae (5–6 days after hatching), pupae and adults (newly emerged) were sampled for the analyses. Aquatic stages (larvae and pupae) were collected from basins using sterile Pasteur pipets and adult mosquitoes from cages using a mouth-suction aspirator.

Field-collected mosquitoes

Wild mosquitoes analysed in this study were sampled from four different areas: 39°51′33″N 18°09′49″E, Torre Mozza, Italy; 11°11′N 4°17′W, Bobo-Dioulasso, Burkina Faso, Africa; 23°52′48″N 90°16′05″E, Dhaka, Bangladesh, Asia; and 22°53′08″S 48°26′42″W, Botucatu, Sao Paulo State, Brazil. Samplings were carried out during the summer of 2014, 2015 and 2016. Adult female mosquitoes belonging to the genera *Anopheles*, *Aedes* and *Culex* (*An. gambiae*, *Cx. quinquefasciatus*, *Culex pipiens*, *Ae. aegypti* and *Ae. albopictus*) were caught using a mouth-suction aspirator, outdoors on human bait and indoors. Once in the laboratories, the collected samples were immediately frozen and processed for DNA extraction in order to avoid contamination with the insectary environments. The identification of the mosquitoes was performed on morphological bases and confirmed by sequencing of the ribosomal gene region ITS2 according to Bachellerie and Qu (1993).

Isolation and identification of yeasts from the reared mosquitoes and insectary water samples

The fungal community present in the gut of adult female mosquitoes was screened in five laboratory reared species by a culture-dependent method. The newly emerged mosquitoes were fed exclusively on sterilized sucrose solution before the dissection of gut. Mosquitoes were carefully washed twice, first in 70% alcohol and then in sterile 1× PBS. Next, guts were dissected using needles under sterile conditions and pooled in 200 μl of NaCl 0.9%. Each pool contained six individual organs per five mosquito species analysed (30 dissected guts), and the assay was replicated on the subsequent mosquito generation (60 total dissected guts). Homogenised pools were pre-inoculated in YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and suspended in saline solution before plating on selective media 6.5% Sabouraud (Sabouraud powder prepared by the manufacturer, BD® Sabouraud Dextrose Agar) with Rifampicin 40 μg/ml. After incubation for 48 h at 28°C, yeast colonies were analysed. PBS recovered from the second washing was plated and examined for the presence of yeast colonies; when no microbes were present in the plates, the mosquito surface sterilization procedure was considered successful.

In addition we examined samples of the water used at our insectary for mosquito rearing. Three different water samples were filtered and analysed: (i) 500 ml of spring water (freshly collected), (ii) 500 ml of breeding water (spring water with sterile mouse food after 2 days exposure to the insectary conditions) and (iii) 500 ml of larval water (breeding water after 5–6 days exposure to the insectary conditions and containing the larval stages of *An. gambiae*). The filters were washed in sterile 1× PBS solution for 30 min. The cells were harvested at 3500 rpm at 4°C and plated on Sabouraud with Rifampicin 40 μg/ml. Plating was replicated twice in independent

assays. After 48 h of incubation at 28°C, the yeast colonies were analysed.

The yeasts were labelled in different groups on the basis of their morphology. To confirm the fungal species, random samples were analysed by restriction fragment length polymorphism (RFLP). The colonies were treated with NaOH (20 mM), boiled at 95°C for 45 minutes (min), and centrifuged at 8000 rpm for 10 min. The supernatants were used as template in PCR reactions in a total volume of 25 μl containing 1 unit Dream Taq and 1× Dream Taq Buffer (Thermo Scientific), 0.25 mM dNTPs, and 0.2 mM oligo yeast-F1 and 0.2 mM oligo yeast-R1. The oligos were designed on conserved sequences of the 18S rRNA gene, amplifying a polymorphic fragment of 796 bp (Ricci *et al.*, 2011a). The cycling conditions began with one step at 95°C for 3 min, followed by 30 cycles of a three-step sequence: 95°C for 30 sec, 50°C for 30 sec and 72°C for 40 sec. The PCR products were digested by restriction enzymes in a total volume of 15 μl containing 1× Buffer Tango and 2 units of both enzymes *Alu1* and *HhaII* (New England BioLabs). The RFLP identified six different patterns, then the sequencing and BLAST analysis (www.ncbi.nlm.nih.gov) of amplified 18S rRNA gene region confirmed the six yeast species.

Setup of the C. parapsilosis*-specific PCR test*

For the set-up of a specific PCR test, two oligonucleotides were designed using the NCBI software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), Cpara-for (5′-ACCC TCGGGTTTGGTGTTG-3′) and Cpara-rev (5′-CCTGA TTTGAGGTCGAATTTGGAAG-3′), amplifying a 132 bp sequence targeting the *C. parapsilosis* ITS2 region. The specificity of the primers was confirmed against the isolated fungal species (*C. parapsilosis*, *M. guilliermondii*, *R. glutinis*, *R. mucilaginosa*, *S.cf. roseus* and *C. haemulonis*) as well as against the mosquito samples. To verify the specificity of the *C. parapsilosis*-specific PCR test, positive amplicons from mosquitoes were analysed by sequencing. The PCR reactions were performed as described above using pure genomic DNAs obtained with the JetFlex Genomic DNA Purification Kit (Genomed, Löhne, Germany). PCR cycling conditions were began with one step at 95°C for 3 min, followed by 30 cycles of a three-step sequence: 95°C for 30 sec, 50°C for 30 sec and 72°C for 40 sec.

Screening for C. parapsilosis *by specific PCR assay in mosquitoes*

C. parapsilosis-specific PCR tests were used to examine both the developmental stages (larvae, pupae and adults) and guts and gonads from male and female adult mosquitoes reared in the insectary. For each species, 12 larvae, 5 pupae and 4 newly emerged adults from three independent experiments were analysed (315 samples), and a different generation of mosquitoes was used in each experimental replicate. Guts and gonads of 6 females and 6 males from three independent experiments were pooled and tested (360 organs analysed). The mosquitoes were reared and processed for the dissection of organs in sterile conditions as previously described.

Concerning wild mosquitoes, a total of 274 field-collected samples were tested. Wild samples were washed as previously described and genomic DNAs were extracted immediately after their transport to the laboratory. The genomic DNA was extracted from individual samples or pool organs, using JetFlex Genomic DNA Purification Kits (Genomed, Löhne, Germany). Quantity and quality of the recovered DNAs were checked by spectrophotometer, before the specific PCR assay.

Results

Screening of the yeast associated with different mosquito species

One hundred and eight yeast isolates were obtained from a total of sixty guts of female mosquitoes reared at our insectary, specifically, *An. gambiae* (45), *An. stephensi* (22), *Ae. albopictus* (22), *Ae. aegypti* (17) and *Cx. quinquefasciatus* (2). Based on the sequenced region of the 18S rRNA gene, four fungal species were identified: *C. parapsilosis*, *M. guilliermondii*, *R. glutinis* and *S. cf. roseus* (Table 1). The overall results indicated *M. guilliermondii* (72/108) and *C. parapsilosis* (33/108) as the most frequently isolated species, whereas *R. glutinis* (2/108) and *S. cf. roseus* (1/108) were detected only sporadically. *M. guilliermondii* was isolated in all the mosquito species analysed. On the other hand, *C. parapsilosis* was the predominant isolate from the female guts of *An. gambiae* but it was not identified in any of the other mosquitoes. The relatively small number of fungal species identified could be due to intrinsic limitations of the culture-dependent method, such as the plating medium used (Rani *et al.* 2009).

Since the insectary environment may impart its own microbes to the mosquitoes, the water samples used for rearing were also examined. Three types of water were analysed: (i) spring water, before exposure to the insectary environment; (ii) breeding water, after 5–6 days exposure in presence of sterile food; (iii) larval water, after 5–6 days exposure in presence of sterile food and *An. gambiae* larvae. One hundred isolates were obtained: spring water (30), breeding water (30) and larval water (40). Based on the sequenced region of the 18S rRNA gene, besides *C. parapsilosis* (31/100) and

Table 1. Fungal species isolated in laboratory reared colonies.

♀ gut (two sets of 6 pooled guts were plated)	No. of fungal isolates	Closest relative	GenBank accession No. (% identity)
Anopheles gambiae	45	***Candida parapsilosis* (33)**	JQ008831.1 (99%)
		Meyerozyma guilliermondii (10)	KM222295.1 (99%)
		Rhodotorula glutinis (2)	HQ420261.1 (99%)
Anopheles stephensi	22	*M. guilliermondii* (22)	KM222295.1 (99%)
Aedes albopictus	22	*M. guilliermondii* (21)	KM222295.1 (100%)
		Sporobolomyces cf. roseus (1)	JN938636.1 (100%)
Aedes aegypti	17	*M. guilliermondii* (17)	KM222295.1 (99%)
Culex quinquefasciatus	2	*M. guilliermondii* (2)	KM222295.1 (100%)
Tot. 60	Tot. 108		

The identification of fungal isolates from the female guts was carried out by sequencing a 796 bp region of the 18S rRNA gene. The closest relative in GenBank and the percentages of identity are reported here. After careful washing of newly emerged mosquitoes (fed on sterilized 5% sucrose solution), guts were dissected and homogenised in pool of six organs per each mosquito species analysed. The analyses were replicated twice on independent assays and the overall data are reported (Tot. 60). No yeasts colonies were developed on control plates used to validate the mosquito surface sterilization procedure.

M. guilliermondii (22/100), two additional yeast species were isolated: *R. mucilaginosa* (42/100) and *C. haemulonis* (5/100). The results indicated *R. mucilaginosa* as the most frequently isolated species, whereas *C. haemulonis* only occurred sporadically (Table 2). Notably, *R. mucilaginosa* was the only fungal species found in the spring water, and its numbers were drastically fewer in the breeding and larval waters; it was not isolated from the gut of any of the mosquito species analysed. Thus, *R. mucilaginosa* could be an environmental contaminant introduced into the insectary from the outside, for instance, since this fungal species was isolated from the pollen (Nogueira *et al*., 2012), it can arrive through the spring water used to raise the mosquitoes. Conversely, *M. guilliermondii* and *C. parapsilosis*, which were detected as the most frequent isolates in the breeding or larval water, respectively, proved to be associated with the insectary environment.

The two culture dependent assays were replicated twice, showing similar trends each time. The overall results indicated that even if the spring water might be a vehicle for fungi to reach the larval gut, this does not necessarily guarantee their association with adult mosquitoes.

C. parapsilosis *PCR-specific tests*

A specific PCR test was carried out to investigate the association of *C. parapsilosis* with mosquitoes. Analyses were performed to examine tissues and developmental stages of mosquitoes from our insectary (*An. stephensi*, *An. gambiae*, *Cx. quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus*), and field collected individuals belonging to several species (*An. gambiae*, *Cx. quinquefasciatus*, *Culex pipiens*, *Ae. aegypti* and *Ae. albopictus*) from sampling sites in four continents.

Regarding the reared mosquitoes, the larvae, pupae, and adults were examined, and for the adult males and females, the guts and gonads were studied. A total of 36 larvae, 15 pupae and 12 adults per species were tested, and the percentages of positive samples are reported in Table 3A. *C. parapsilosis* was detected in

Table 2. Fungal species isolated from the rearing water at insectary.

Water samples	No. of fungal isolates	Closest relative	GenBank accession No. (% identity)
Spring water (i)	30	*Rhodotorula mucilaginosa*	KP233783.1 (98%)
Breeding water (ii)	30	*M. guilliermondii* (20)	KM222295.1 (99%)
		R. mucilaginosa (6)	KP233783.1 (99%)
		***C. parapsilosis* (4)**	JQ008832.1 (99%)
Larval water (iii) (*An. gambiae*)	40	***C. parapsilosis* (27)**	JQ008832.1 (99%)
		R. mucilaginosa (6)	KM222296.1 (100%)
		Candida haemulonis (5)	JN941107.1 (100%)
		M. guilliermondii (2)	KM222295.1 (99%)
	Tot. 100		

The identification of fungal isolates from the water samples was carried out by sequencing a 796 bp region of the 18S rRNA gene. The closest relative in GenBank and the percentages of identity are reported here. Three water samples were analysed: (i) spring water (before exposure at the insectary), (ii) breeding water (after 5–6 days exposure at rearing conditions in presence of sterile larval food) and (iii) larval water (after 5–6 days exposure at rearing conditions in presence of sterile larval food and *An. gambiae* larvae). The analyses were replicated twice on two independent assays and the overall data are reported.

Table 3. Detection of *C. parapsilosis* in the developmental stages and organs by specific PCR assay.

Mosquito species (Unicam strains)	Developmental stages (A) (12 larvae, 5 pupae, 4 adults were tested individually in triplicate)			Organs (B) (Three sets of 6 pooled organs from adult mosquitoes were tested)			
				Gut		*Gonad*	
	Larvae	*Pupae*	*Adults*	♀	♂	♀	♂
An. gambiae	97% (35/36)	73% (11/15)	67% (8/12)	+	+	+	+
An. stephensi	97% (35/36)	100% (15/15)	100% (12/12)	+	+	+	+
Cx. quinquefasciatus	92% (33/36)	73% (12/15)	25% (3/12)	+	+	−	−
Ae. aegypti	67% (24/36)	53% (8/15)	25% (3/12)	+	+	+	+
Ae. albopictus	100% (36/36)	93% (14/15)	100% (12/12)	+	+	+	+

(A) **Developmental stages**: The percentages of *C. parapsilosis*-positive samples are reported per species analysed. The tests were performed in three generations of mosquitoes. For each generation, 12 larvae, 5 pupae and 4 newly emerged adults were analysed (tot. 315 individuals). (B) **Organs**: Guts and gonads from newly emerged mosquitoes were analysed as 6 pooled organs in three generations of mosquitoes (tot. 360 organs).

three generations of reared colonies, confirming its persistent occurrence in mosquitoes, and its presence was documented in all three developmental stages. In the larvae, the results showed 67% positivity in *Ae. aegypti* and from 92% to 100% in the other mosquito species. Interestingly, the percentages of positive individuals decreased from larvae and pupae to adults in *An. gambiae* (67%), *Cx. quinquefasciatus* (25%) and *Ae. aegypti* (25%), whereas they remained constant in *An. stephensi* and *Ae. albopictus*.

We focused further on the adult female and male gut and gonads (Table 3B). The PCR assays detected *C. parapsilosis* in the gut and gonads of all the species analysed, except in the gonads of *Cx. quinquefasciatus*.

In order to investigate the presence of *C. parapsilosis* in the wild, we tested different species of field-collected samples from Italy, Africa, Asia and Brazil captured in the summer of 2014, 2015 and 2016 (Table 4). We tested 274 female adult mosquitoes, finding positivity to *C. parapsilosis* in all the groups analysed: *An. gambiae* (35/60), *Cx. quinquefasciatus* (5/20), *Culex pipiens* (22/36), *Ae. albopictus* (43/88) and *Ae. aegypti* (41/70). The data indicated that *C. parapsilosis* was present in all the species and all four locations, with 53% of samples proving positive.

Discussion

The four fungal species identified in the mosquito gut have been described as pathogens of clinical significance. *C. parapsilosis* is one of the most common isolates from bloodstream infections and *M. guilliermondii* is known as an opportunistic pathogen isolated from clinical specimens (Papon et al., 2013; Tan *et al.*, 2016). *R. glutinis* and *S. cf. roseus* are both reported, albeit rarely, as opportunistic pathogens (McNicholas et al., 2012; Menon *et al.*, 2014). Moreover, all the fungi isolated in this study share similar metabolic and antimicrobial traits that might suggest possible symbiotic associations with mosquitoes.

Interestingly, insects may represent reservoirs of yeasts that are somehow involved in the host's

Table 4. Detection of *C. parapsilosis* in field-collected mosquitoes.

Mosquito species (wild strains)	No. of positive/No. of examined field-collected individuals	Sampling sites
An. gambiae (n. 60)	35/60	Bobo-Dioulasso, Burkina Faso
Cx. pipiens (n. 36)	16/30	Bobo-Dioulasso, Burkina Faso
	6/6	Torre Mozza, Italy
Cx. quinquefasciatus (n. 20)	5/20	Dhaka, Bangladesh
Ae. aegypti (n. 70)	15/30	Botucatu, Sao Paulo State
	26/40	Dhaka, Bangladesh
Ae. albopictus (n. 88)	29/36	Torre Mozza, Italy
	11/17	Botucatu, Sao Paulo State
	3/35	Dhaka, Bangladesh
	Tot. 146/274	

Adult female mosquitoes were collected from the field in four sampling sites during 2014–2016. African sampling site (2015): Bobo-Dioulasso (11°11′N 4°17′W), Burkina Faso; Italian sampling site (2014): Torre Mozza (39°51′33″N 18°09′49″E), Italy; Asian sampling site (2015): Dhaka (23°52′48″N 90°16′05″E), Bangladesh; Brazilian sampling site (2016): Botucatu, Sao Paulo State (2016). The detection of *C. parapsilosis* was carried out by specific PCR test.

nourishment and/or defence. An example is the killer yeast *Wickerhamomyces anomalus*, isolated from beetles, sand-flies and several mosquito species, where it exerts important functions for the development or the antimicrobial protection of the host (Ricci *et al*., 2011b; Toki *et al*., 2012; Cappelli *et al*., 2014; Martin *et al*., 2015; Valzano *et al*., 2016). Notably, our findings in laboratory reared colonies supported a previous study in wild *Culex* sp. that documented the cohabitation of such killer yeasts, as *C. parapsilosis*, *M. guilliermondii*, *Rhodotorula* sp. and *W. anomalus* in the mosquito (Steyn *et al*., 2015).

R. glutinis was described for its killer activity against phytotoxic fungi (Castoria *et al*. 2005) and several *Rhodotorula* sp. with *S. cf. roseus* were found in soft scales, where they may favour the growth of the host developmental stages (Zacchi and Vaughan-Martini, 2003). *M. guilliermondii*, which was identified in plant-associated beetles (Suh *et al*., 2008), mosquitoes (Frants and Mertvetsova, 1986; Steyn *et al*., 2015) and sand-flies (S. Epis, unpublished data), exerts antimicrobial activities (De Lima *et al*., 2013) and the ability to over-synthesise the vitamin B2 (Protchenko *et al*., 2000) that could be useful during the pupal starvation. *C. parapsilosis* was reported as an antimicrobic in biocontrol applications (Niknejad *et al*., 2012) and documented for lipase activity (Yalçın *et al*., 2014). Thus, like *M. guilliermondii*, its defensive/nutritional symbiosis in insects could be suggested. Notably, *C. parapsilosis* was observed as the only fungal species shared between adults and larvae of wild *Cx. pipiens* from a near-by pond, thus escaping the microbial reduction/elimination during the development of larvae to adult (Steyn *et al*., 2015). This finding suggests a possible effect of *C. parapsilosis* on mosquito ontogeny, a role that was already suggested for *W. anomalus* in beetles (Toki *et al*., 2012). Further analyses are needed to verify the function of *C. parapsilosis* in the host as reported for other killer yeasts.

Considering the potential role of some insects in spreading killer yeasts that may also act as opportunistic pathogens in anthropized environments, these kinds of associations are worthy of major investigation. Our study on *C. parapsilosis* documented a stable yeast-mosquito association through three laboratory reared generations examined. Its presence was detected in pre-adult and adult stages in all the colonies analysed even if with different positive percentages in larvae, pupae and adults depending on the mosquito species. Notably, a diverse localisation of *C. parapsilosis* was detected in the mosquito organs: it was present in all the guts analysed and in the gonads of all the species analysed except *Cx. quinquefasciatus*. Interestingly, the colonization of the gonads suggests that some transovarial transmission may occur in some mosquito species.

Finally, we reported a screening on the circulation of *C. parapsilosis* in field collected samples from Italy, Africa, Asia and Brazil, finding a wide diffusion of the yeast in the wild mosquitoes. The data confirmed the yeast association observed in laboratory reared samples also for wild *An. gambiae*, *Cx. quinquefasciatus*, *Ae. albopictus* and *Ae. aegypti*. To our knowledge, this is the first report of *C. parapsilosis* in these species. Moreover, the yeast detection in *Cx. pipiens* from Burkina Faso and Italy confirmed the presence of the yeast in additional wild populations besides those analysed from South Africa by Steyn and collaborators in 2015.

Based on these findings, we suggest that mosquitoes might afford an additional habitat and a suitable environmental reservoir for *C. parapsilosis*, participating in its environmental spreading not just through a simple passive dissemination.

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